Table 1. Total uptake of [^{14}C]glyphosate from commercial formulations when applied to velvetleaf

Hours after treatment (HAT)	[^{14}C]Glyphosate uptake[a] (% of applied close)($\pm$SE)		
	Roundup Ultra	Roundup	Touchdown
3	18.6 ($\pm$1.4)	8.5 ($\pm$0.7)	2.8 ($\pm$0.5)
6	35.4 ($\pm$1.8)	21.9 ($\pm$0.9)	4.9 ($\pm$0.4)
24	40.7 ($\pm$2.3)	35.9 ($\pm$1.5)	13.9 ($\pm$1.3)
72	39.4 ($\pm$2.6)	32.3 ($\pm$1.1)	25.6 ($\pm$1.5)

[a] Mean of six determinations.

treated leaf is a function of uptake minus the export into the plant. Radioactivity analysis of treated leaves showed rapid loading by Ultra, followed by Roundup and Touchdown.

The amount of glyphosate translocated away from the treatment site into the plant is believed to be a key indicator of efficacy. Glyphosate translocation was very fast with Ultra, followed closely by Roundup, with that from Touchdown being considerably slower. At 72 HAT, translocation of glyphosate with Ultra was more than twice that with Touchdown (19% vs 9% of applied dose). Although leaf loading of glyphosate from Ultra and Roundup reached a maximum by 6 HAT, the translocation of glyphosate did not reach a maximum until 24 HAT. This is evidence that the treated leaf represents an intermediary pool which serves as the source of glyphosate export into the phloem.

Using [^{2}H]NMR, we developed a simple method for measuring leaf permeability to water. Glyphosate is expected to be in close association with water, so that the impact of surfactants on leaf permeability to water should also translate to glyphosate. The objective was to determine whether deuterium oxide might be used as a marker for glyphosate in plant uptake studies. The experiment involved pre-treating the leaf with diluted glyphosate formulation (12.5 g AI litre^{-1}) followed by application of deuterium oxide to the same drop sites. The control plant received the formulation and deuterium oxide at the same time. In order to reduce biological variability, diluted Roundup (12.5 g AI litre^{-1}), used as the internal control, was applied to one-half of the leaf and the second formulation (Ultra or Touchdown) was applied to the other half of the same leaf. Leaves were pre-treated with the formulations for 6 h, followed by a 30-min deuterium oxide treatment time. Relative to Roundup, Ultra and Touchdown showed 197% and 68% deuterium oxide leaf penetration, respectively. These results correlate with the uptake of [^{14}C-]glyphosate from these formulations.

The examination of three commercial formulations of glyphosate has identified distinct patterns of glyphosate uptake and translocation, and of leaf damage. We observed a direct correlation between the speed and the extent of uptake with leaf damage. Tissue damage within 24 h after application was caused by the surfactant, based on examination of blank formulations containing no glyphosate. Our results suggest that surfactant tissue damage plays a key role in penetration of glyphosate into the leaf.

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Ecdysone agonists – Mechanism of action and application on *Spodoptera* species

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Abstract: Laboratory assays using artificial diet demonstrated that tebufenozide (RH-5992) and the new structural analogue, RH-2485 (proposed common name methoxyfenozide), possess strong ecdysone-like activity against last-instar larvae of the beet armyworm, *Spodoptera exigua*, and the cotton leafworm, *Spodoptera littoralis*, leading to precocious lethal moulting. LC_{50} values showed that the activity of RH-2485 (0.38 mg AI litre^{-1}) was about twice that of tebufenozide (0.60 mg AI litre^{-1}) in *S. exigua*, whereas in *S. littoralis* respective LC_{50} values were 1.15 mg AI litre^{-1} and 9.51 mg AI litre^{-1}. The retention-fate curves of ^{14}C-radiolabelled ecdysone agonist could not explain the differential toxicity values between species and compounds. Ingestion of the oxidase inhibitor piperonyl butoxide (PB) synergized the toxicity of the ecdysone agonist, indicating the importance of oxidative detoxifcation in *Spodoptera* larvae, and may raise the possible use of PB as synergist for this group of insecticides, or for monitoring resistance due to increased oxidation.

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1 INTRODUCTION

The beet armyworm, *Spodoptera exigua* (Hübner), and the cotton leafworm, *Spodoptera littoralis* (Boisduval), are two key polyphagous noctuid pests of worldwide importance that feed on various agricultural crops, including vegetables, cotton and ornamentals. In various places, resistance has been reported over the last two decades after extensive use of broad-spectrum insecticides, leading to failure or reduced efficacy of insecticides such as chlorinated hydrocarbons, organophosphates (OPs), carbamates and pyrethroids. Likewise, there have been reports of low efficacy of benzoylphenyl ureas and *Bacillus thuringiensis* Berliner formulations in some areas.

Tebufenozide (RH-5992) and the novel structural analogue, RH-2485 (proposed common name methoxyfenozide), are synthetic non-steroidal agonists of the insect moulting hormone, manifesting their effects, especially in Lepidoptera, via interaction with the ecdysteroid receptors.[1–6] Hence, compounds of this new group of insect growth regulators (IGRs) with their novel mode of action may be suitable for integrated pest and resistance management programs for the control of important lepidopteran pests, such as *Spodoptera* species.

The objectives of this research were to determine the activity of tebufenozide and RH-2485 against last-instar larvae of *S. exigua* and *S. littoralis*, in combination with their pharmacokinetic fate in the larval body of the two species. The synergistic effects of piperonyl butoxide (PB), a potent inhibitor of oxidative enzymes, were evaluated and discussed.

2 EXPERIMENTAL METHODS

2.1 Insects

All stages of *S exigua* and *S littoralis* were kept at standard conditions of 23(±2)°C, 70(±5)%RH and a 16L:8D photoperiod.[4]

2.2 Bioassays

2.2.1 Larvicidal toxicity tests

Toxicity assays with tebufenozide and RH-2485 (Rohm and Haas, Spring House, PA, USA; AgrEVO, Diegem, Belgium) were performed with newly moulted (0–12 h after ecdysis) last-instar larvae.[3] Mortality counts were made when control larvae had metamorphosed into one-day-old pupae, and the data were then subjected to probit analysis using the POLO-PC program.[7] Activity was evaluated as LC_{50} values (FL_{95}) and slopes (±SE); POLO-PC uses a χ^2 test at $P = 0.05$ to detect significant differences.

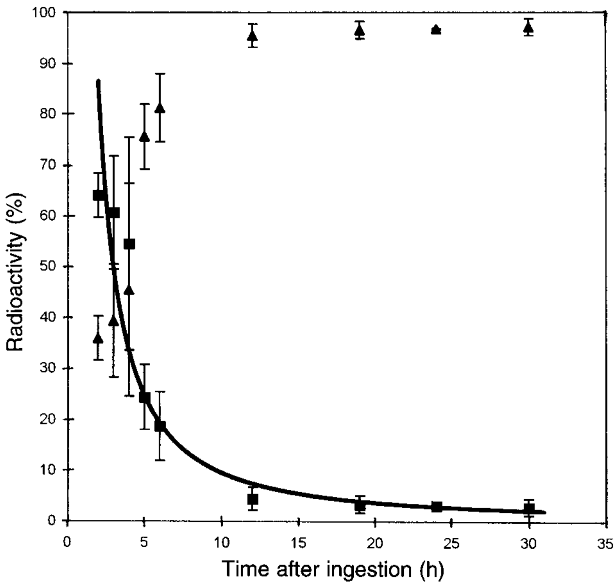

Figure 1. [^{14}C]Radioactivity (■) in the body and (▲) faeces of newly moulted (0–2 h) last-instar larvae of *Spodoptera exigua* after ingestion of a leaf disc treated wih [^{14}C]tebufenozide. Data are means of three replicates.

2.2.2 Synergism assay

Technical piperonyl butoxide (PB) (Fluka, Bornem, Belgium) was tested as a synergist of tebufenozide and RH-2485. Ecdysone agonist was provided to newly moulted (0–12 h) last-instar larva alone or with PB as a uniform layer covering the surface of the artificial diet.[8] No mortality was scored when last-instar larvae were fed PB-treated diet up to the highest concentration used (100 mg litre^{-1}). Percentages of mortality were corrected according to Abbott for the mortality of the untreated control.[9]

2.3 Fate of tebufenozide and RH-2485

Newly moulted (0–2 h) last-instar larvae were selected and individually starved for 6 h in a 4.5-cm diameter Petri dish. A solution of ^{14}C-*tert*-butyl] tebufenozide (specific activity 85.32×10^7 Bq g^{-1}, Rohm and Haas Ltd) in methanol (1 µl) was applied to a freshly cut disc from a castor bean leaf (*Ricinus communis* L); *S exigua* larvae received 6066(±776) and *S. littoralis* larvae 43302(±5981) dpm per leaf, respectively. [^{14}C-*tert*-butyl]*RH*-2485 (specific activity 85.32×10^7 Bq g^{-1}; Rohm and Haas Co) was applied at 7389(±1774) dpm. After complete consumption of the leaf disc, three replicate sets of two last-instar larvae were selected at different time intervals and stored in a freezer at −20°C until analysis. Uptake of radioactivity in the larval body and excretion via the faeces were determined using a Biological Material Sample Oxidiser (Packard).[10]

3 RESULTS AND DISCUSSION

3.1 Larvicidal activity

After treatment with tebufenozide or RH-2485, intoxicated larvae showed signs of premature and

lethal moulting within one day of feeding on treated diet. The old head capsule slipped down, and a fragile and non-sclerotized new head capsule was observed underneath the old capsule: larvae died without shedding the old cuticle. Simultaneously, feeding and weight gain of such treated larvae were significantly suppressed (data not shown). This effect agrees with observations in other insects.[1–6] Similar results have been reported following application of natural ecdysteroids,[11] indicating a state of hyperecdysonism.[12] Altogether, the current symptoms confirm the moulting hormone-mimicking mode of action of both non-steroidal compounds.

Based on LC_{50} values in *S exigua*, RH-2485 was 1.58 times and in *S littoralis* about eight times more active as a result of oral feeding with treated diet than tebufenozide (Table 1). The current activity of tebufenozide and RH-2485 agree with the findings of other authors,[2–4,6,13] suggesting that RH-2485 is a potent compound for controlling caterpillars.

3.2 Fate of tebufenozide and RH-2485

Retention pharmokinetics for [^{14}C]tebufenozide and [^{14}C]RH-2485 in the larval body of *S exigua* after uptake of a treated leaf disc (Fig 1) followed eqns (1) and (2), respectively:

$$Y = 223.5X^{-1.4} \qquad (R^2 = 0.95) \tag{1}$$

$$Y = 113X^{-1.3} \qquad (R^2 = 0.77) \tag{2}$$

where Y is the percentage radioactivity in the larval body and X is the time after ingestion (h). From this it is apparent that the excretion rate $[Y'(X)]$ of RH-2485 was somewhat higher than that for tebufenozide, especially in the first hours after treatment. At 6 h after ingestion, about 20% of the radioactivity of tebufenozide and 10% of that of RH-2485 remained in the body. Six hours later, these values had decreased to about 7% for both compounds. In the last instar of *S littoralis*, [^{14}C]tebufenozide amounted to 33% at 6 h after ingestion and 8% after a further 6 h.

In general, the current uptake and retention profiles agree with the fate of substituted dibenzoylhydrazines that could be measured in other insects.[2,10] It has been shown that these compounds have, in general, a relatively high metabolic stability and are excreted rather quickly as parent compound after absorption in the body tissues. The data obtained so far may suggest a faster excretion of RH-2485 in the faeces via the gut as compared to tebufenozide, resulting in a relatively lower absorption in the body tissues. So, the higher potency of RH-2485 may result from factors other than retention such as a better translocation and higher metabolic stability within the larval body tissues, and a higher affinity to bind to the ecdysteroid receptor.

3.3 Synergism by piperonyl butoxide

In a series of oral feeding assays, we determined the ability of PB to synergise the toxicity of tebufenozide and RH-2485 in *S exigua* and *S littoralis* larvae, thus evaluating the importance of oxidative metabolism towards tebufenozide and RH-2485. In *S exigua*, addition of PB enhanced the toxicity of RH-2485, showing a synergism ratio (SR) of 1.69-fold at the highest concentration of PB. Addition of 5, 20 and 100 mg PB litre^{-1} to a concentration of 0.4 mg AI litre^{-1} RH-2485 resulted in mortality of 68, 73, and 91%, respectively, as compared to 54% mortality with RH-2485 alone. In a recent study we have reported that simultaneous application of tebufenozide and PB resulted in an LC_{50} of 0.18 mg litre^{-1} as compared to 0.58 mg litre^{-1} with tebufenozide alone (SR of 3.4).[8] In larvae of *S littoralis*, concentrations of 5 and 10 mg AI litre^{-1} tebufenozide applied together with 100 mg AI litre^{-1} PB resulted in 100 and 92% mortality, as compared to 0 and 60% mortality when the ecdysone agonist was applied alone (Fig 2). Another group was offered diet treated with 1 mg litre^{-1} RH-2485 only and in combination with 5, 10 and 100 mg litre^{-1} PB. The latter two combinations scored 100% mortality, leading to a synergism activity of at least 2.4-fold.

It is a well-known phenomenon that an increased metabolic activity in insects is an important mechanism of resistance to various compounds. So the synergistic effect of PB, an inhibitor of oxygenases, with tebufenozide and RH-2485 suggested that oxidases are important in the detoxification process. Thus, we hypothesize that the major first-phase route of detoxification for tebufenozide and RH-2485 is through oxidation, but to a different extent in the two species. Mixture with PB at a ratio of 1:5 in *S exigua* larvae indicated that RH-2485 (SR = 1) shows a higher metabolic stability than tebufenozide (SR = 3.4). In

Table 1. Oral toxicity of tebufenozide and RH-2485 against last-instar larvae of *Spodoptera exigua* and *Spodoptera littoralis*[a]

Species	*Product*	*LC_{50} (mg litre^{-1})*[b]	*Slope (±SE)*
S exigua	Tebufenozide	0.60 (0.56–0.65)	13.1 (±0.6)
	RH-2485	0.38 (0.31–0.45)	7.0 (±1.6)
S littoralis	Tebufenozide	9.51 (8.18–11.14)	4.7 (± 0.5)
	RH-2485	1.15 (0.79–1.50)	4.1 (±0.5)

[a] Toxicity based on mortality percentages scored at 24 h after ecdysis of the control larvae. Data are based on a minimum of seven different doses, subjected to probit analysis with POLO-PC.[7]
[b] 95% fiducial limits in parentheses.

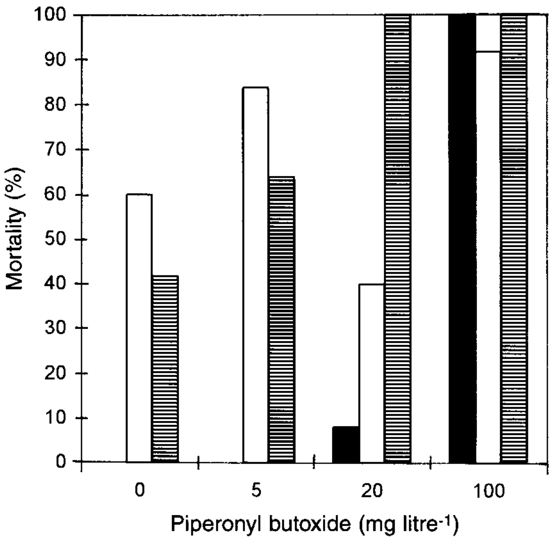

Figure 2. Effect of different concentrations of piperonyl butoxide (PB), on the activity of tebufenozide at (■) 5 mg litre^{-1} and (□) 10 mg litre^{-1}, and (▤) RH-2485 at 1 mg litre^{-1} against newly moulted (0–12 h) last-instar larvae of *Spodoptera littoralis*. Scores are corrected using Abbott's formula for control mortality.

S litoralis, addition of PB at a ratio of 1 : 5 could increase the toxicity of 1 mg litre^{-1} RH-2485 from 41 to 64% (SR = 1.56), and the SR value for tebufenozide with and without PB at 100 mg AI litre^{-1} was 1.53, suggesting that oxidation might be of a similar importance for both compounds. However, further biochemical metabolism and enzyme studies are required to establish a firm conclusion.

The results obtained thus far indicate that PB can be a useful tool to increase the toxicity of ecdysone agonists and to screen for enhanced oxidase levels in various resistant specimens. P_{450} oxidases are important in resistance development towards other groups of insecticides such as pyrethroids, OPs and carbamates.[14] Hence, cross-resistance with other insecticides should be carefully analysed. These lines of research are in progress in our laboratory and are aimed at providing guidelines for resistance management of this group of insecticides in the future.

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